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Identification of methotrexate transport deficiency in mammalian cells using fluoresceinated methotrexate and flow cytometry

(antifolate uptake/dihydrofolate reductase/methotrexate resistance)

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ABSTRACT **We have studied the frequency of transport mutations in methotrexate-resistant Chinese hamster ovary cells using a rapid-flow cytometric technique. After saturating cells with fluoresceinated methotrexate, we examined the ability of hydrophilic and lipophilic antifolates to displace fluoresceinated methotrexate binding to dihydrofolate reductase. Cells with methotrexate transport deficiency are unable to take up methotrexate and thus retain the fluorescence, whereas the lipophilic antifolates displace fluoresceinated methotrexate equally well in sensitive and resistant cell lines. These resistant clones fail to take up methotrexate and occur with high frequencies upon single-step selections at methotrexate concentrations ≈7-fold the 50% killing concentration. The majority of such first-step resistant clones appear to derive their resistance solely from transport deficiency; they exhibit no overproduction of dihydrofolate reductase and no increase in either steady-state mRNA levels or gene copy number. Possible applications of the use of fluoresceinated methotrexate to the characterization of various mechanisms of methotrexate resistance in mixed cell populations are discussed.**

The 4-amino analog of folic acid, methotrexate (MTX), is a potent inhibitor of dihydrofolate reductase (DHFR) from a variety of sources (1). Mammalian cells acquire resistance to continuous exposure to MTX by one or more mechanisms, including (*i*) increased levels of the target enzyme as a result of *DHFR* gene amplification (2), (*ii*) altered affinity of DHFR for MTX (3, 4), and (*iii*) reduced permeability to the drug by virtue of defects in a carrier-mediated transport system (5–7). These classes of resistance may occur independently or together in individual cell variants (8).

Just as the use of flow cytometry and fluoresceinated MTX (F-MTX) derivatives (9) has been useful in studying MTX resistance resulting from overproduction of DHFR as a result of gene amplification (10, 11), the use of such techniques would be most useful for studying that class of MTX resistance resulting from altered (defective) inward transport. Rosowsky *et al.* (12) have suggested that the reduced uptake of a fluorescent lysine analog of MTX (PT430) may directly reflect an alteration of inward MTX transport. However, since MTX at high concentrations only partially competed with PT430 uptake, the possibility that MTX and PT430 have different transport routes could not be ruled out.

We have found that F-MTX penetrates Chinese hamster ovary cells via a mechanism that is distinct from the carrier-mediated transport of MTX (unpublished data), as suggested earlier for L1210 mouse leukemia cells (13). We here describe a flow cytometric assay in which F-MTX is used as an indirect probe for the identification of MTX-resistant cells exhibiting defective inward MTX transport. The assay takes advantage of the fact that F-MTX binding to DHFR can be displaced by MTX in sensitive cells but is retained in cells with a transport defect. We have applied this technique to an analysis of the frequency and mechanism(s) of resistance that occur upon selection of MTX-resistant colonies at a single concentration of MTX ≈7 times that which is the 50% killing concentration of normal cells.

MATERIALS AND METHODS

Drugs. MTX was obtained from the National Cancer Institute and aminopterin was purchased from Sigma. Metoprine and BW301U {2,4-diamino-6-(2,5-dimethylbenzyl)-5-methylpyrido[2,3α]pyrimidine} were kindly provided by C. Nichol (Welcome Research Laboratories, Research Triangle Park, NC). F-MTX was purchased from Molecular Probes (Eugene, OR).

Cell Lines and Cultures. Chinese hamster ovary (CHO) AA8 cells (14) were maintained under monolayer or suspension conditions in minimal essential medium (MEM) supplemented with 10% of either whole or dialyzed fetal bovine serum (GIBCO) containing gentamicin (GIBCO). CHO K1B11 0.5 cells stably resistant to 0.5 μM MTX (15) and CHO DG-44 (a DHFR-deficient variant; see ref. 16) were grown as specified in the references. Cells in monolayer were passaged in exponential phase by standard trypsinization, while suspension cells were diluted into fresh medium.

Plating Efficiencies and Single-Step Selection with MTX. AA8 cells were plated (2.5×10^3–10^5 cells per 10-cm dish) in medium containing 0.15 μM MTX and an additional amount of 2 mM glutamine. Eleven to 13 days later, resistant colonies were picked at random (1 clone per dish) using cloning rings. For colony counts, cells were washed with phosphate-buffered saline (PBS), fixed with methanol, and stained with crystal violet. The plating efficiency of AA8 cells in normal growth medium was >95%.

F-MTX Labeling and Flow Cytometry. Cells from the midexponential phase (7 cell doublings after plating) were incubated with 2 μM F-MTX for 8 hr in folate-deficient MEM supplemented with dialyzed fetal bovine serum, glycine, hypoxanthine, and thymidine (30 μM). Cells were washed with PBS, trypsinized, resuspended in PBS, and analyzed with a Coulter Epics 753 flow cytometer as described (17).

DNA and RNA Blot Hybridization. Cells were lysed, underlayered with a sucrose cushion, and separated into nuclear and cytoplasmic fractions by centrifugation. High molecular weight DNA and cytoplasmic RNA were isolated from the respective fractions as described (18). *DHFR* gene copy number and mRNA levels were estimated by Southern blot (after digestion with *Eco*RI) and RNA blot analyses, using standard capillary transfer (19) and by the slot-blot method (20) using a ^{32}P oligolabeled plasmid (21) containing the 3′-most exon of the functional hamster *DHFR* gene (22),

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Abbreviations: DHFR, dihydrofolate reductase; MTX, methotrexate; F-MTX, fluoresceinated methotrexate; BW301U, 2,4-diamino-6-(2,5-dimethylbenzyl)-5-methylpyrido[2,3α]pyrimidine.

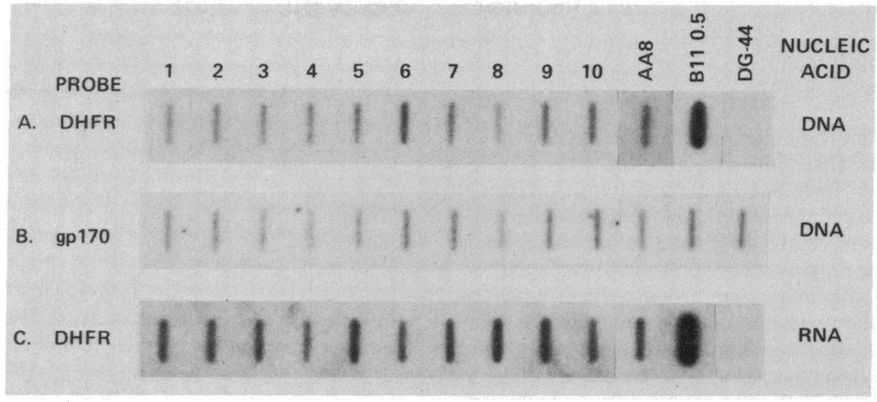

FIG. 1. *DHFR* gene copy number and mRNA levels in MTX-resistant clones. Ten CHO AA8 clones resistant to 150 nM MTX, parental sensitive cells (AA8), and B11 0.5 as well as a DHFR-deficient cell line (DG-44) were grown to 5×10^7 cells. Nucleic acids were isolated and 2 μg of DNA or 10 μg of cytoplasmic RNA (0.2 μg of DNA and 1 μg of RNA for B11 0.5) was immobilized on Zetabind filter paper and hybridized to a ^{32}P oligolabeled hamster DHFR probe (rows A and C). DNA (2 μg) was also probed with a hamster cDNA for the *MDR1* gene as a control (row B). Filters were hybridized at 42°C for 48 hr and washed under high stringency conditions (18 mM NaCl/1 mM $NaPO_4$, pH 7.7/0.1 mM EDTA/0.1% $NaDodSO_4$ at 67°C for 2 hr).

and a hamster cDNA clone of the *MDR1* (multidrug resistance) gene (23) as a control.

Cell Extracts and DHFR Enzyme Assays. Cells from exponential phase grown in the absence of MTX for six cell doublings were detached by trypsinization, washed three times with PBS, and counted. Cells were lysed in 0.1 M phosphate buffer (pH 7.5) (10^7 cells per ml), sonicated for 1–2 min, and centrifuged at 15,000 × *g* for 15 min at 4°C. The supernatant was stored at −80°C. Folate reductase activity was assayed by the [^{3}H]folic acid reduction assay (24). Blank values were determined using cell extracts from DHFR-deficient CHO cells. Protein content was determined by the Bio-Rad protein assay (25). [^{3}H]MTX (specific activity, 250 mCi/mmol; 1 Ci = 37 GBq; Amersham) and [^{3}H]folate (specific activity, 54.5 Ci/mmol; Amersham) were purified prior to use by high-performance liquid chromatography on a column of octadecylsilane (250 × 4.6 mm, Alltech, Deerfield, IL) as described by Kamen *et al.* (26). The purified compounds were concentrated under vacuum, dissolved in 10% ethanol, and stored at −80°C to retard radiolysis (7). Their concentrations and specific activities were determined spectrophotometrically using appropriate extinction coefficients (27) and by scintillation counting.

Transport Measurements. Exponentially growing cells in suspension (1–2 × 10^5 cells per ml) in MTX-free MEM/dialyzed fetal bovine serum medium were harvested by centrifugation and washed in PBS, and the uptake of [^{3}H]MTX was measured as described (6).

RESULTS

Frequency of MTX Resistance. Subjecting AA8 CHO cells to a single-step selection with 0.15 μM MTX results in a frequency of resistant colonies in fetal bovine serum-supplemented medium varying between 2.7 and 6.2 × 10^{-4}, while in medium supplemented with dialyzed fetal bovine serum the frequency was 2.5–3.4 × 10^{-5}. We attribute this difference to the removal of purine, glycine, thymidine, and perhaps reduced folates from the serum by dialysis and thereby generating a more stringent selection.

***DHFR* Gene Copy Number and mRNA Levels.** MTX-resistant variants were cloned and analyzed for *DHFR* gene copy number and for mRNA levels. Only 5 of the 22 clones examined have evidence of *DHFR* gene amplification, which was at most a 2 to 3-fold amplification. In Fig. 1, we show 10 of the clones that were selected for further characterization. None of these clones showed any increase either in *DHFR* gene copy number or in steady-state mRNA levels. Scanning densitometry of the autoradiograms from different experiments confirmed the fact that the majority of single-step MTX resistances were not associated with increases in either *DHFR* gene copy number or mRNA content (Table 1). The 10 clones described retained the MTX-resistant phenotype when grown in the absence of MTX for 40 cell doublings. The B11 0.5 cells showed a 40- to 60-fold increase in gene copy number and mRNA levels as shown previously (15). Thus, in confirmation of prior studies on single-step (low concentration) MTX selections with mouse 3T6 cells, a variable proportion of the cell variants have no amplified *DHFR* genes, and those that do have small gene copy increments (20). With no MTX-resistant variants did we detect any gene rearrangement phenomena by either Southern blot or RNA blot analyses.

DHFR Enzyme Levels in MTX-Resistant Variants. DHFR enzyme levels in 10 MTX-resistant clones were determined by measuring enzyme specific activity and by flow cytometric analysis of cells saturated with F-MTX. Table 1 shows

Table 1. Summary of relative *DHFR* gene copy number, mRNA, and enzyme levels of MTX-resistant clones

	AA8	B110.5	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10
DHFR gene copy number	1	50.4	0.7	0.8	0.8	0.8	1.0	1.3	1.0	0.7	1.2	1.3
MDR1 gene copy number	1	1.3	0.8	0.8	0.9	0.9	0.7	1.0	0.9	0.7	1.1	1.1
DHFR mRNA levels	1	48.5	0.9	0.9	0.7	0.5	1.3	0.7	1.0	1.3	1.5	0.8
DHFR specific activity*	1	115	1.1	1.2	1.3	1.2	1.5	1.3	0.9	1.0	1.6	1.2
F-MTX labeling†	1	6.1	1.1	1.1	1.3	1.1	1.0	0.9	1.5	1.1	1.2	1.0

Results are expressed as mean values relative to parental sensitive cells (AA8). Values in this table are the average from two to five experiments. The maximal variation between experiments was ≈15%.

*The mean specific activity of sensitive cells was 3.4 ± 0.4 units per mg of protein.

†Values are corrected for autofluorescence (of unstained cells), which was ≈15% fluorescence after saturation with F-MTX staining.

that the relative specific activities of the enzyme (measured as folate reductase), as well as the values for F-MTX binding, were essentially similar to sensitive cell values, while the B11 0.5 cells showed elevated values by these two parameters of DHFR activity.

Competition of F-MTX Binding with Various Antifolates in Sensitive and Resistant Cells. The addition of 0.1 μM MTX to sensitive cells after they have been saturated with F-MTX (2 μM) for 8 hr results in a rapid loss of fluorescence of cells with a half-time of 1 hr, whereas in MTX-resistant cells displaying no evidence of *DHFR* gene amplification no loss (displacement) of F-MTX occurs (Fig. 2). Thus, we propose that impaired transport of MTX in the resistant cells may account for their inability to displace cellular F-MTX from binding to DHFR. To examine this proposal, we have compared the loss of fluorescence in sensitive and resistant cell variants during incubation with various concentrations of MTX or its structural homolog aminopterin. As shown in Fig. 3, the concentrations of MTX and aminopterin necessary to compete out 50% of F-MTX binding in 3 hr were 30 and 6.6 nM, respectively, whereas in MTX-resistant cells (clone 10) the 50% displacement concentrations were much higher (1500 and 1380 nM, respectively). Since the hydrophilic folate analogs MTX and aminopterin failed to compete with bound F-MTX in MTX-resistant cells over a wide concentration range, we also examined the ability of the lipophilic antifolates metoprine and BW301U (28) to compete with bound F-MTX in these cells. Fig. 4 shows that these lipophilic antifolates efficiently compete for F-MTX binding in both sensitive and resistant cells. Table 2 shows that the 10 independently isolated MTX-resistant clones share the property of retention of F-MTX staining when challenged with MTX but lose all of F-MTX staining when exposed to metoprine.

Uptake of [^{3}H]MTX in MTX-Resistant Variants. To provide direct evidence that the resistance in these single-step selected clones not displaying evidence of *DHFR* gene amplification was the result of impaired MTX transport, [^{3}H]MTX was purified and transport measurements were undertaken.

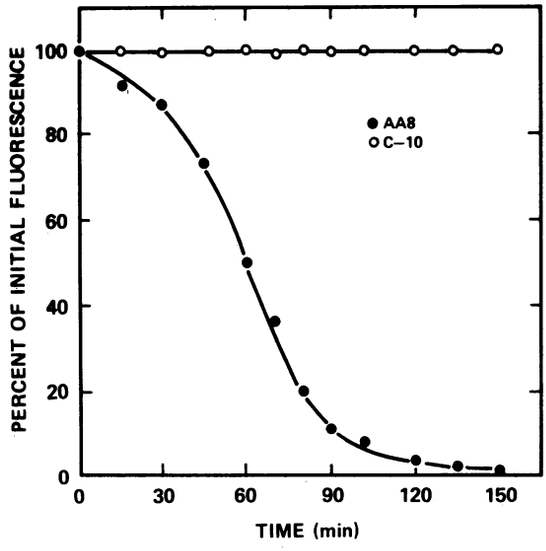

FIG. 2. Time-dependent competition of F-MTX staining of sensitive and MTX-resistant cells with MTX. Sensitive or MTX-resistant cells (clone 10) were saturated with 2 μM F-MTX for 8 hr and then the medium was replaced by medium containing 0.1 μM MTX. At 15-min intervals cells in monolayer were washed, detached, and analyzed for mean fluorescence per cell by flow cytometry. Each point represents the percentage of remaining net fluorescence (after subtraction of autofluorescence of unstained cells) compared to the initial value. The forward-angle light scatter was essentially the same in both MTX-resistant and sensitive cells, which indicates similarity in cell size.

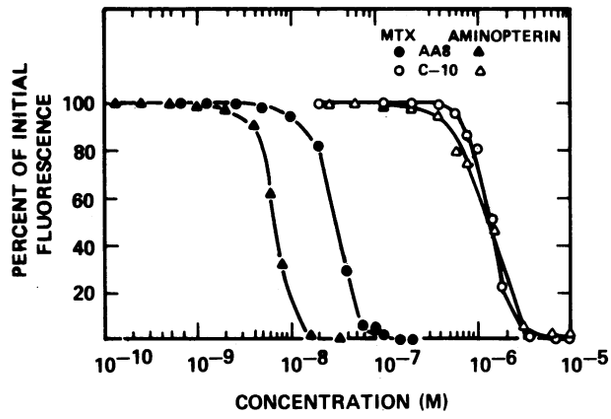

FIG. 3. Competition of F-MTX labeling with MTX and aminopterin. After staining of sensitive and MTX-resistant cells with F-MTX, MTX or aminopterin was added to the medium at various concentrations. After incubation for 3 hr at 37°C, the percentage of F-MTX remaining was calculated, based on the fluorescence of stained cells without any addition of MTX or aminopterin for the 3-hr incubation period.

Fig. 5 shows that at an external concentration of 0.5 μM essentially no time-dependent uptake of MTX was detected in three independently isolated MTX-resistant variants, whereas the parental AA8 cells had a typical time-dependent saturation uptake curve with an initial rate of 6.1 pmol per 10^8 cells per min. These results suggest that these clonal variants derive their resistance to MTX solely from an impaired inward transport of MTX.

DISCUSSION

To characterize rapidly mechanisms of MTX resistance in heterogeneous cell populations, whether they are derived in the laboratory or occur clinically, it would be highly desirable to have a method that is less laborious than the cloning of individual cell variants. We have previously used a fluoresceinated derivative of MTX and flow cytometric techniques to study various properties of cultured cells with amplified *DHFR* genes. Here we describe the use of F-MTX to identify that class of MTX resistance resulting from defective inward MTX transport, a technique that makes use of the fact that F-MTX appears to penetrate CHO cells by a mechanism distinct from the carrier-mediated transport of MTX (unpublished data) as suggested by Henderson *et al.* (13). Thus, we

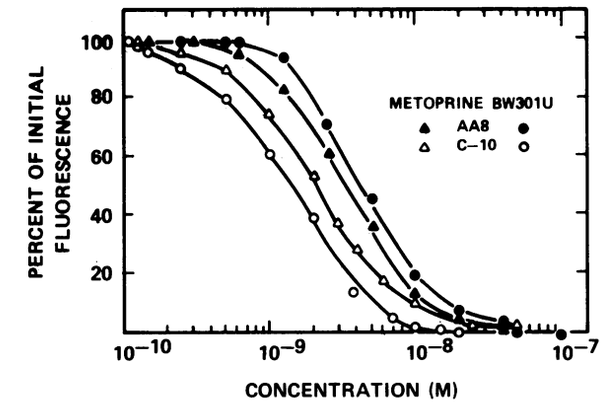

FIG. 4. Competition of F-MTX labeling with metoprine and BW301U. Cells stained with F-MTX as described in Fig. 3 were incubated in medium containing various concentrations of metoprine or BW301U and the percentage of remaining fluorescence was determined as described in Figs. 2 and 3.

Table 2. Retention of F-MTX staining in resistant cells after the addition of antifolates

Antifolate	AA8	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10
MTX											
0.1 μM	0	97	95	99	97	100	98	100	98	97	98
1.0 μM	0	68	66	87	69	81	75	69	82	74	75
5.0 μM	0	0	0	0	0	0	0	0	0	0	0
Metoprine											
10 nM	14	8	5	12	4	16	8	23	10	16	10
100 nM	0	0	0	3	0	5	0	12	1	1	2

Cells were saturated with 2 μM F-MTX for 8 hr and then incubated for 3 hr in various concentrations of MTX or metoprine. Values are expressed as the percentage of remaining cellular fluorescence in the presence of the competing antifolate (average of three experiments).

find that cells resistant to MTX with impaired inward MTX transport are labeled with F-MTX as efficiently as sensitive cells. However, because of a defective transport system, the F-MTX bound to DHFR is not displaced by subsequent incubation of resistant cells in MTX, whereas in the parental sensitive cells the fluorescence is rapidly displaced. Consistent with the conclusion that the displacement requires a functional MTX carrier system is the fact that lipophilic folate analogs (metoprine and BW301U) displace F-MTX equally well in both sensitive and resistant cell lines.

Flow cytometric methods using F-MTX may provide a relatively simple means of assessing cell populations for mixed mechanisms of MTX resistance. Thus, the degree of increased fluorescence is generally a reflection of DHFR gene amplification. However, the caveat of such a conclusion is the possibility that a mutation may reduce the affinity of MTX and F-MTX for DHFR even in the presence of extensive *DHFR* gene amplification (see, for example, refs. 4 and 8). Transport-defective MTX-resistant variants can be identified on the basis of whether MTX can displace the internally bound F-MTX. We have preliminary indications that the CHO B11 0.5 cell line with amplified *DHFR* genes also is transport defective by the MTX-displacement criteria. Perhaps this is not surprising, given the high frequency of transport resistances and the length of time this cell line has been maintained in MTX with the opportunity for mutational events producing altered transport. Gaudray *et al.* (11) have reported that some MTX-resistant variants fail to take up F-MTX despite measurable DHFR activity and have suggested that this discrepancy is the result of impaired uptake of F-MTX. Inasmuch as impaired uptake of MTX can involve a number of varieties of alterations in the carrier transport system (5), including absence of carrier protein, altered V_{max} or K_m, etc., the use of flow cytometric techniques to distinguish between different types of transport-defective phenotypes may be more difficult, in particular in mass cell cultures or heterogeneous tumor cell populations.

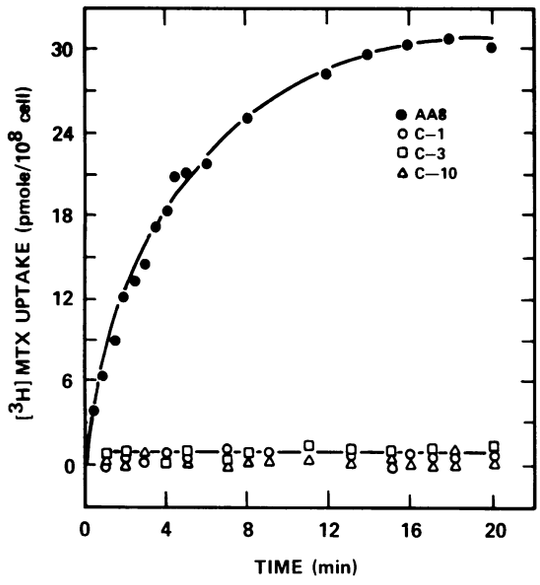

FIG. 5. Time course of [^{3}H]MTX accumulation in sensitive and MTX-resistant cells. Logarithmically growing cells were washed three times with PBS, adjusted to 5×10^7 or 1×10^8 cells per ml in 0.15 M Hepes buffer (pH 7.3) containing 1 mM $MgCl_2$ and incubated with shaking at 37°C. Uptake was initiated by adding [^{3}H]MTX to 0.5 μM. At various intervals 0.1-ml aliquots of cell suspensions were removed, placed in 15 vol of ice-cold PBS, and sedimented at 15,000 × *g* for 30 sec. Cells were resuspended in 100 vol of PBS and centrifuged. Cells in the pellet were lysed with 0.5% Triton X-100/10 mM Tris·HCl, pH 7.5/1 mM EDTA and quantitatively transferred, and radioactivity was determined by liquid scintillation counting.

Although the amplification of *DHFR* genes has received much attention, altered MTX transport is also frequently observed, particularly in drug-resistant cells obtained *in vivo* in mouse and human tumors (5, 29). When cultured cell lines are step-selected for MTX resistances to MTX concentrations far beyond any that could be sustained *in vivo*, we have always observed *DHFR* gene amplification. This is understandable from the fact that at high—i.e., nontherapeutic—concentrations, MTX can enter cells by a mechanism(s) that circumvents the carrier-mediated process, most likely by passive diffusion. Thus, in culture, a transport-defective cell variant would not survive multiple step selections without, in addition, *DHFR* gene amplification. We have, indeed, observed this type of phenomena with a rat hepatocyte cell line (30). We are currently interested in the type of mutational event(s) leading to defective transport, since various treatments of mouse and CHO cells that enhance the frequency of *DHFR* gene amplification (in first-step selection protocols) also enhance equally extensively the frequency of another resistance mechanism(s). This enhancement may be as much as 10^3-fold greater than control frequencies (20, 31, 32). Our current studies suggest that transport defects may be a major form of such enhanced resistances that are not gene amplification events.

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